AUTHENTICATION OF β-CARBONIC ANHYDRASE GENE SILENCING IN *DROSOPHILA MELANOGASTER*

Henna Niemelä

Bachelor's Thesis
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Degree Programme in
Laboratory Science

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ABSTRACT

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Authentication of β-Carbonic Anhydrase Gene Silencing in *Drosophila melanogaster*

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One widely used organism in the field of genetic research is *Drosophila melanogaster*, the fruit fly. *Drosophila* can be considered as an ethically acceptable model organism. Moreover, it is easy to grow and the progeny development is fast and plentiful. For instance, different kinds of loss-of-function studies can be performed using fruit flies, such as silencing of the β-carbonic anhydrase gene. Carbonic anhydrases control the acid-base regulation as well as many other physiological pathways in different organisms. β-class carbonic anhydrases can be found in plants and invertebrates but not in vertebrates.

The objective of this Bachelor's thesis was to generate a fruit fly population in which β-carbonic anhydrase gene could be silenced by using a hormone containing media, and to authenticate the silencing with different approaches. The purpose was to establish the methods for authentication and to identify optimal hormone concentration for gene silencing.

To achieve silencing, RNA interference and UAS-GAL4 -systems were used with hormone based media. The gene expression levels were studied with quantitative polymerase chain reaction. In addition, immunoblotting was also conducted to examine the protein level changes, and histological samples were studied to find out the occurrence of β-carbonic anhydrase in the fly tissue.

Alterations in gene expression were discovered although the data obtained from qPCR were not altogether consistent. The immunoblotting results suggest that the antibody used did not function as desired. Histological stainings were adjusted and improved, but the findings are challenging to evaluate. In the future it would be desirable to cross different fly stocks and increase the sample amount. The studies will continue with methods established in this work.

Key words: carbonic anhydrase, *Drosophila melanogaster*, gene silencing, PCR, GAL4, RNAi.

TIIVISTELMÄ

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NIEMELÄ, HENNA:

β-hiilihappoanhydraasi -geenin hiljennyksen todentaminen *Drosophila melanogasterissa*

Opinnäytetyö 54 sivua, joista liitteitä 6 sivua

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Drosophila melanogaster on laajalti käytetty malliorganismi geneettisten sairauksien tutkimisessa, sillä useat ihmisen tautigeenit löytyvät tältä selkärangattomalta vastingeeneinä. Banaanikärpästä voidaan pitää eettisesti hyväksyttävänä mallieläimenä, jonka ylläpito on helppoa ja jälkeläismäärät suuria. Hiilihappoanhydraasit taas esiintyvät eri muodoissa eukaryooteissa ja prokaryooteissa. Nämä entsyymit osallistuvat organismin pH-tasapainon säätelyyn ja myös muihin tärkeisiin fysiologisiin prosesseihin. β-hiilihappoanhydraasia esiintyy pääasiassa selkärangattomissa ja kasveissa.

Opinnäytetyön tavoitteena oli eri *D. melanogaster* -kantoja risteyttämällä saada aikaan populaatio, jonka β-hiilihappoanhydraasi -geeni on mahdollista hiljentää lääkeruoan avulla. Tarkoituksena oli tutkia missä lääkeruokapitoisuudessa geeni hiljentyy, sekä etsiä ja pystyttää sopivia menetelmiä hiljentymisen todentamiseen.

RNA interferenssiä ja GAL4-UAS -menetelmää apuna käyttäen saatiin aikaan hiljennyksessä käytettävä jälkeläissukupolvi. Hiljentymistä tutkittiin geenin ekspression kautta kvantitatiivisella polymeraasiketjureaktiolla. Hiljentyminen pyrittiin myös osoittamaan proteiinitasolla western blot -menetelmällä ja histologisten värjäysten avulla pyrittiin toteamaan β-hiilihappoanhydraasin esiintyminen kudoksissa.

Viitteitä β-hiilihappoanhydraasi -geenin hiljentymisestä saatiin geenitasolla, joskin tulokset eivät olleet täysin johdonmukaisia. Immunoblottaus -menetelmästä saatujen tulosten mukaan käytetty vasta-aine ei toiminut täysin toivotusti. Histologisia värjäyksiä saatiin kehitettyä, joskin tuloksia hiljentymisestä on vaikea tulkita. Tutkimuksia jatketaan tutkimusryhmässä työssä pystytettyjä menetelmiä ja saatuja tuloksia apuna käyttäen. Tulevaisuudessa hiljentymistä voitaisiin tutkia eri kärpäslinjoilla ja suuremmalla näytemäärällä.

Asiasanat: Hiilihappoanhydraasi, *Drosophila melanogaster*, geenin hiljentäminen, PCR, GAL4, RNAi.

LIST OF CONTENTS

ABBREVIATIONS

βCA	Beta-Carbonic Anhydrase
CA	Carbonic Anhydrase
DAB	3,3'-diaminobenzidine tetrahydrochloride
DCR	A subunit of DCR/R2D2 -protein complex
DEPC	Diethylpyrocarbonate
ECL	Enhanced chemiluminescence
GAL4	Transcription activator protein from yeast
GS	GeneSwitch
HE	Haematoxylin-eosin -staining
HRP	Horseradish peroxidase
MIF / RU486	Mifepristone
NTC	Non-template control
PAGE	Polyacrylamide gel electrophoresis
PVDF	Polyvinylidene fluoride
R2D2	A subunit of DCR/R2D2 -protein complex
RISC	RNA induced silencing complex
RNAi	Ribonucleic acid interference
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylethylenediamine
UAS	Upstream Activation Sequence

1 INTRODUCTION

Many pathways in different organisms are dependent on precise pH as for instance enzymes work only in their optimal pH -range. The regulation of acid - base balance is therefore a profound mechanism within every organism, representing a physiologically important phenomenon where carbonic anhydrases are involved. Many pathways can be studied in a widely used model organism, *Drosophila melanogaster*, by for example knocking down genes of interest. The fruit fly models have been widely used and there are numerous transgenic fly stocks available for research.

The objective of this study was to silence β-carbonic anhydrase gene expression by using RNA interference and GAL4-UAS -method with GeneSwitch -hormone receptor in different *D. melanogaster* crossings. Consequently, the purpose was to conclude in which hormone concentration the silencing occurs and constitute three different methods for authentication of the gene silencing.

The experimental part of the Bachelor's Thesis was performed in the University of Tampere in Professor Seppo Parkkila's Tissue Biology research group. The research group has characterized many factors relating to carbonic anhydrases and their occurrence in different tissues.

2 REVIEW OF THE LITERATURE

2.1 Carbonic anhydrases

The first carbonic anhydrases (CAs) were purified from red blood cells in 1933, as the original studies gave strong evidence that carbonic anhydrases are enzymes (Meldrum & Roughton 1933, 140-141; Stadie & O'Brien 1933, 528). By catalyzing the rather simple reaction: $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$, where carbon dioxide is hydrated into bicarbonate and a proton, carbonic anhydrases regulate the acid-base balance in organisms (Sly & Hu 1995, 380). The active element in several CAs contains a zinc ion (Zn^{2+}), which is fundamental for catalysis (Supuran, RE 2008, 168).

Carbonic anhydrases are an important group of universal metalloenzymes which are present in all organs and implicate in diverse physiological and pathological pathways. For instance, CA-regulated processes involve lipogenesis (Bray 1972, 546), bone resorption and osteoporosis (Sly et al. 1983, 2753-2756), gluconeogenesis (Herbert, Coulson & Hernandez 1982, 190-191) and transport of CO_2 and HCO_3^- (Longmuir, Foster & Woo 1966, 393-394). In recent times it has become clear that CA inhibitors or activators may be used as potential antiglaucoma, anti-obesity, anticancer and anti-infective drugs (Supuran, RE 2008, 168).

2.1.1 β-carbonic anhydrase

CAs can be classified into five different groups, α, β, γ, δ and ζ -classes which differ from each other at the level of their tertiary and quaternary structures. Classes α and γ consist strictly of monomers and trimers as in β-class can be found dimers, tetramers, hexamers and octamers. (Liljas & Laurberg 2000, 16; Kimber & Pai 2000, 1408-1410.) These classes can be identified everywhere in the phylogenetic tree, for instance, α-class CAs are mainly found in vertebrates and β-class in plants, archae and eubacteria (Chegwidden & Carter 2000, 13).

Structural studies have suggested that βCA class significantly differs from other classes not only in total protein folding but also in catalytic mechanisms. Figure 1 demonstrates

that the βCA structure has two domains alpha and beta containing four zinc molecules. β-class has been found in algae, higher plants and prokaryotes (Mitsuhashi et al. 2000, 5521, 5526) but as well in a variety of invertebrates, such as fruit flies, nematodes and mosquitoes though they are not presented in chordates (Syrjänen et al. 2010, 8).

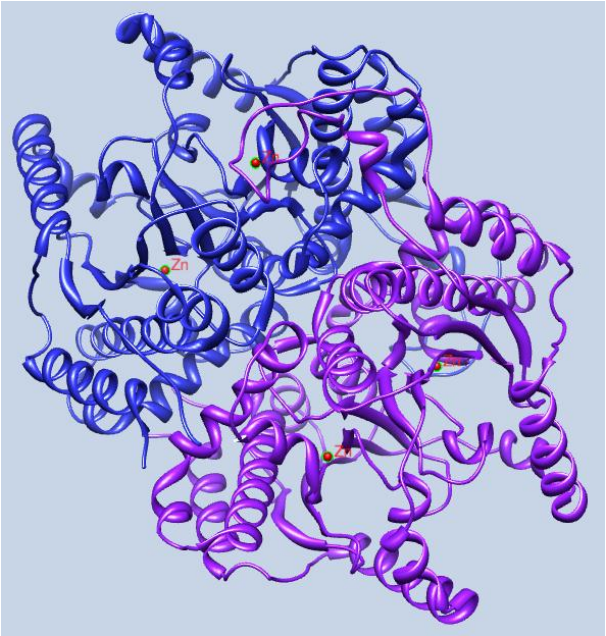

FIGURE 1: β-carbonic anhydrase consists of two domains, alpha (upper) and beta (lower) and active sites' four Zn molecules are found in the middle of the domains. (PDB ID: 1DDZ).

Despite the differences between the classes all carbonic anhydrase enzymes catalyse the same reaction where a water molecule is activated by a zinc ion resulting in reaction with carbon dioxide or as in reverse reaction destabilizing bicarbonate (Liljas & Laurberg 2000, 16).

2.2 *Drosophila* as a model organism

Drosophila melanogaster is a small fruit fly which has proven to be one of the most valuable organisms in the field of biological research. *Drosophila* models have been used as a genetic tool for over 100 years, since the days of T.H. Morgan, the pioneer of fruit fly studies. Morgan proved the chromosome theory to be correct by crossing *Drosophilas*. (Morgan 1910, 120-122.)

Drosophila melanogaster females can produce hundreds of eggs and the interval between generations is short: in +25 °C degrees the development from egg to fly takes about ten days. *D. melanogaster* tolerates a relatively wide range of environmental surroundings and stock lines are easy to culture, although the stocks require frequent maintenance. (Ashburner, Golic & Hawley 2005, 17, 122-123.)

This easy to handle invertebrate has helped to reveal and aid human disorders hence over 75 percent of human disorder genes are implicated in *Drosophila* (Reiter et al. 2001, 1114). Alzheimer's disease (Torroja, Chu, Kotovsky & White 1999, 491-492), Huntington's disease (Jackson et al. 1998, 636-637) and Amyotropic Lateral Sclerosis commonly referred to as ALS (Parkes et al. 1998, 171-174) are only a few examples of studied *Drosophila* proteinopathies. Because fruit flies are invertebrates they can be considered as ethically acceptable model organisms, as animal testing should be carried out by using the organism whose central nervous system is the most primitive available. (FINLEX 2006).

2.2.1 Drosophila melanogaster

The individuals of *D. melanogaster* tend to be light brown. The most evident differences between males and females are that females tend to be bigger and the black striping differs between the sexes in the abdomen. Abdomen is darker in the male, and males have black sex combs, which are used in mating, in their forelegs. (Ashburner, Golic & Hawley 2005, 1256; Weigmann et al. 2003, 310.)

At +25°C degrees the lifecycle of *D. melanogaster* is the following: shortly after mating the female lays fertilized eggs on the media, and about 23 hours later, larvae hatch from the egg. There are three larval stages called the 1st, 2nd and 3rd instars. At first, the larvae consume the media surface, then burrow into the media and finally after around 100 hours of feeding, arise from the food for pupariation. The pupariation follows after approximately 120 hours after egg laying and it lasts for about 4 days after which adult flies emerge. (Ashburner et al. 2005, 122.) Three developmental stages of the white eyed *D. melanogaster's* complete metamorphosis are illustrated in figure 2 where a larvae, a pupa and an adult female fly can be seen.

FIGURE 2: The development of white eyed w1118 stock fly from larvae to pupa into an adult female fly.

To ensure that the parents of the planned cross are the ones desired, female fruit flies are collected as virgins, since mated female flies can store sperm from males in their ventral receptacle and spermathecae. Virgins are collected 8–10 hours after eclosion. (Ashburner et al. 2005, 123, 134-135.) Virgin flies can be distinguished from adults as they are more pale (Markow & O'Grady 2006, 217) and a dark spot, fly's meconium, can be seen in the translucent abdomen (Greenspan 2004, 21).

2.2.2 RNA interference

Ribonucleic acid interference (RNAi) as a phenomenon was found accidentally by plant biologists in the early 1990's (Napoli, Jorgensen & Lemieux 1990, 286-287). The following studies with a nematode *Caenorhabditis elegans* revealed the actual mechanisms behind RNAi and earned a Nobel Prize in Physiology or Medicine for its discoverers. (Fire et al. 1998, 806-807; The Nobel Foundation 2012).

RNAi is a method in which addition of sequence specific double stranded RNA (dsRNA) causes the break down of corresponding mRNA resulting in inhibition of translation and gene silencing. Gene silencing by dsRNA occurs basically in the same way in all eukaryotes, from *Drosophilas* to humans, through diversified pathway - though the enzymes and proteins are named differently from one species to another. (Tropp 2008, 810-812.) Transgenic *Drosophila* lines are created by carrying the transgenic RNAi constructs into flies by for example using viral particles, plasmids or by injection. Yet these dsRNAs, such as small hairpin micro RNAs (shmiRNA), small

hairpin RNAs (shRNA), synthetic small interfering RNA (siRNA) or dsRNA, have limited operating time without tissue specificity. (Perrimon, Ni & Perkins 2010, 2, 4.) The effects are also dose dependent (Ashburner et al. 2005, 385).

In the *Drosophila* pathway, initiation happens by a steady heterodimer protein. The first subunit of which belongs to RNase III enzyme group is named Dicer (DCR). The other subunit is called R2D2 and it is an RNA binding protein. Actually, in the *Drosophila* pathway there are two different DCR homologs: DCR1 responsible for miRNA production and DCR2 which produces siRNAs. (Liu et al. 2003, 1921-1925; Lee et al. 2004, 69-81.) As figure 3 depicts (p. 12), DCR-R2D2 binds to dsRNA in cytoplasm hydrolyzing it into small RNA molecule, 21-28 bp long microRNAs (miRNAs) or siRNAs. These small RNAs have 2-3 nucleotide overhangs at 3' -ends and in 5' -end phosphate groups attached. (Tropp 2008, 810-812; Snustad & Simmons 2010, 604-606.)

Ribonucleoprotein complex attaches to a small RNA molecule unwinding the double strand leaving only one strand left (Snustad & Simmons 2010, 604-606). This strand of small RNA is combined with RNA induced silencing complex (RISC) which catalyzes target mRNA disintegration. The formation of RISC requires the preliminary stage of RISC loading complex which contains the heterodimer, small RNA duplex, and other yet uncharacterized proteins. (Tropp 2008, 810-812.)

If perfectly base paired, the impact between small RNA molecule attached to RISC and the complementary sequence in the target mRNA prevents the expression of the gene by siRNAs. This happens by RNA hydrolysis as RNA-protein complex is directed to the center of target mRNA, ten nucleotides from 5' -end of the small RNA. If mRNA and the small RNA molecule cannot base pair perfectly no cleavage happens but translation is blocked by miRNAs. RISC acts like catalyst thus it can operate repeatedly without losing its degrading features. (Snustad & Simmons 2010, 604-606.) RNAi can be used with expression systems such as GAL4-UAS, to express a dsRNA construct resulting in gene silencing in a temporally and spatially controlled manner (Ashburner et al. 2005, 385).

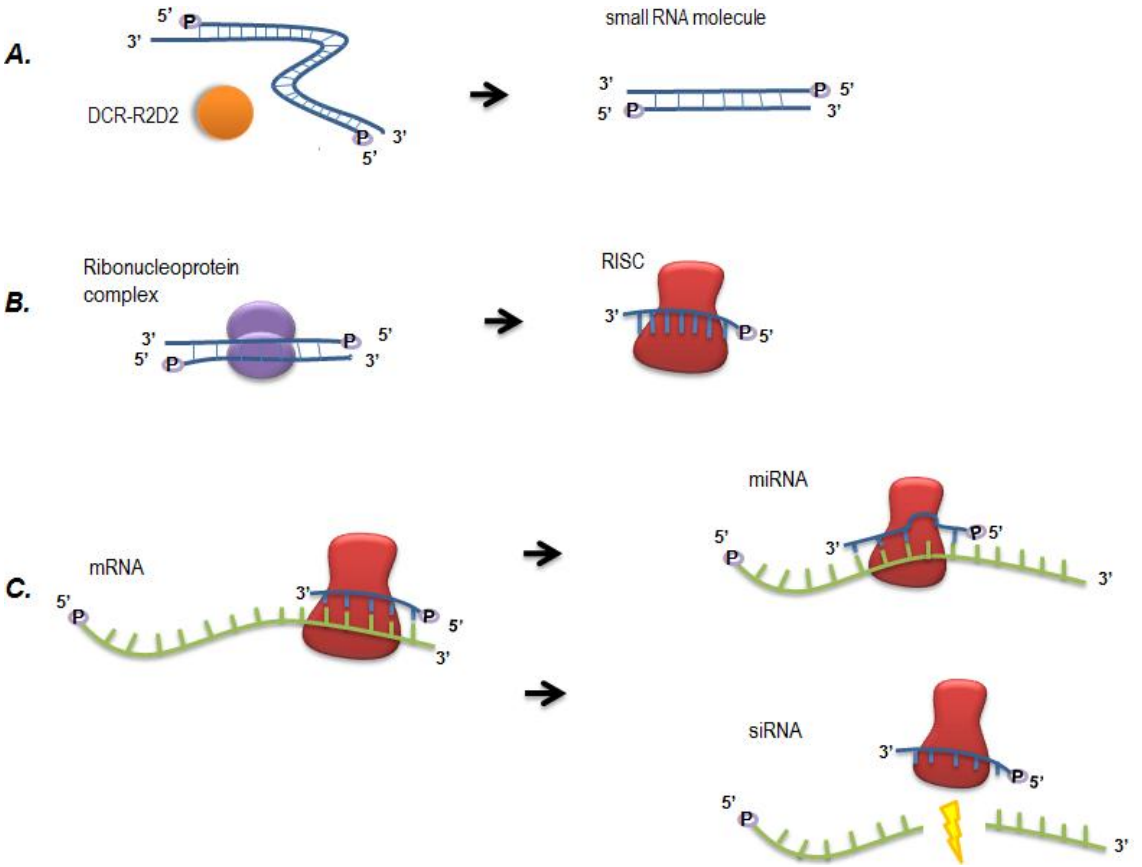

FIGURE 3: RNAi pathways in *D. melanogaster*. (A.) dsRNA is hydrolyzed into small RNA molecules by DCR-R2D2 -complex. (B.) Ribonucleoprotein -complex attaches to small RNA and unwinds it to a single strand for RISC. (C.) RISC guides the small RNA to target mRNA. (Based on Tropp 2008.)

2.2.3 The GAL4-UAS system

The ability to control gene expression in a designated fashion is a useful tool for examining its effects in development. By using Brand's & Perrimon's (1993, 401-415) GAL4-UAS -method specific gene expression can be controllably altered.

The GAL4 is a transcription factor which encodes a protein of 881 amino acids. It has been identified in *Saccharomyces cerevisiae* as an activator of genes controlling galactose metabolism. (Laughon & Gesteland 1984, 263-264.) The GAL4 protein is a dimer with two transactivation domains and it attaches to DNA with zinc finger binding to four 17 -base pair -sites located in the upstream activation sequence (UAS). (Elliot & Brand 2008, 80.)

In this bipartite system shown in figure 4 (p. 14), genes which are controlled by UAS can be activated by tissue specific GAL4 protein. Silencing happens when a certain sequence producing dsRNA is inserted near UAS. The GAL4-UAS -system relies on two separate fly lines: in the first line, the gene or dsRNA producing fragment inserted downstream of UAS (UAS-gene X) remains silent as it has no activator, whereas in the second fly line the activator protein GAL4 is present but has no target gene to activate. (Elliot & Brand 2008, 79.) This enables viable parental lines (Brand & Perrimon 1993, 403) and furthermore the GAL4 can activate transcription in other species, even distantly related, because of the high level of conservation in the eukaryotic transcriptional machinery (Elliot & Brand 2008, 80).

The activity of the GAL4 protein is temperature dependent: in *Drosophila* the ideal GAL4 activity can be reached at +29°C degrees without usually affecting fly viability or fertility (Duffy 2002, 3). Controlled gene silencing can be produced by the GAL4-UAS method which produces specific dsRNA. The dsRNA is hydrolyzed to small interfering RNAs which prevent mRNA translation. (Perrimon, Ni & Perkins 2010, 4.)

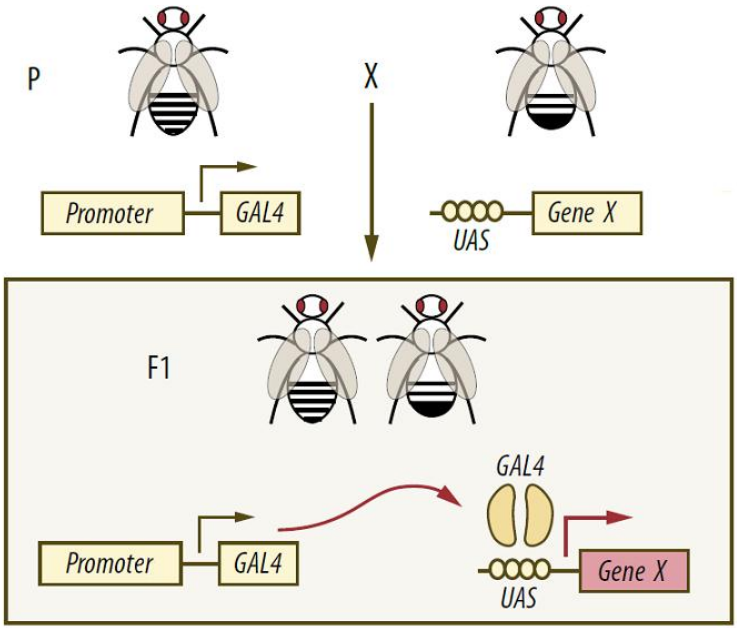

FIGURE 4: The GAL4-UAS -system in *D. melanogaster*. (Adapted from Valanne & Rämet 2011, 2066)

2.2.4 GeneSwitch

Mifepristone (RU486) is a synthetic steroid hormone which has a potent antiprogesterone activity as it blocks progesterone receptor. It is commonly used as abortifacent, inducing chemical abortion in women. (Saeb-Parsy et al. 1999, 263.) RU486 can also be used in regulating gene expression in hormone inducible variant of GAL4 (Roman, Endo, Zong & Davis 2001, 12605-12607; Osterwalder, Yoon, White & Keshishian 2001, 12599-12601).

Gene activity can be regulated in *Drosophila* at most developmental stages by using GAL4 -progesterone receptor activation domain GeneSwitch. Exposing hormone receptor -based GAL4-UAS -progeny to RU486 results in targeted gene expression. (Han, Stein & Stevens 2000, 573, 580-582; Osterwalder et al. 2001, 12599-12601.) The transcriptional activation in GAL4-progesterone receptor requires the proper ligand otherwise no transcription occurs. The onset of the expression happens by feeding the ligand to larvae or flies. The reaction is a reversible as removing the larvae or flies from the presence of ligand stops the transcription. (Elliott & Brand 2008, 84.)

GeneSwitch is expressed with its enhancer in GAL4 driver fly line, which is crossed with UAS -responder line having genomic insertion of a target gene as illustrated in figure 5. Without activator GeneSwitch protein is expressed but remains inactive and cannot bind to UAS in the responder line. When RU486 is available, the GAL4 protein becomes active and is able to bind to UAS. Therefore expression of the responder sequence is activated in tissues expressing GeneSwitch. In addition, gene expression levels are dependent on the dosage of RU486 concentration in medium. (Osterwalder et al. 2001, 12596-12601.)

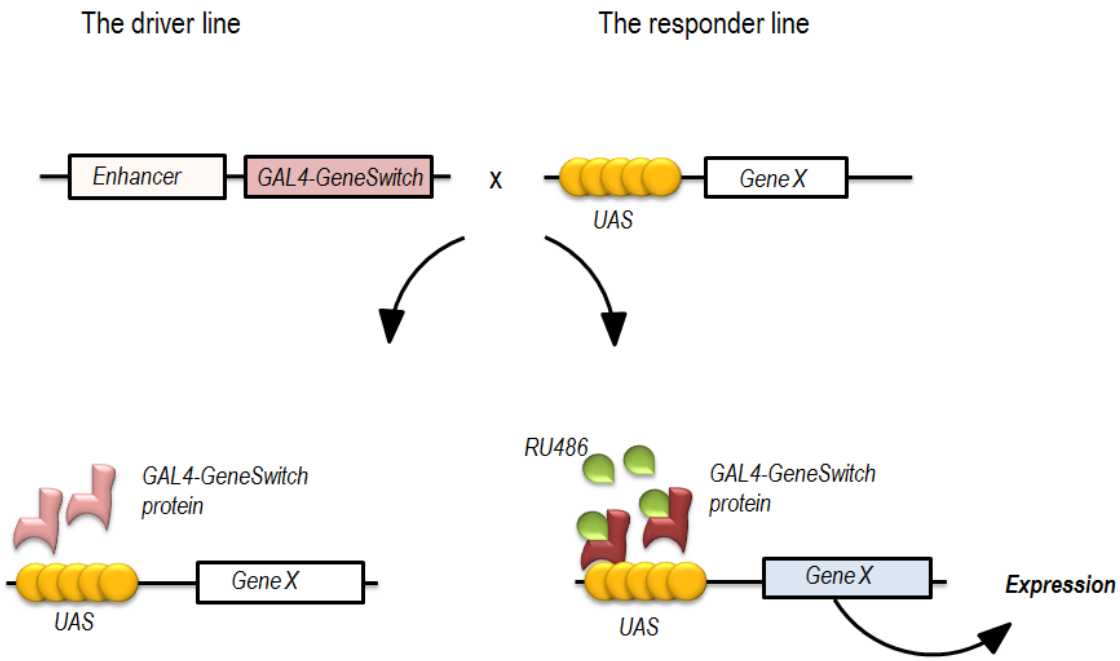

FIGURE 5: GAL4-UAS-progesterone -receptor. In the absence of the ligand (RU486) GAL4-GeneSwitch -protein encoded by the GAL4-GeneSwitch region, remains inactive. When RU486 is present the protein becomes active and the gene is expressed, for instance to produce certain dsRNA. (Based on Duffy 2002.)

3 AUTHENTICATION METHODS OF SILENCING

3.1 Quantitative real-time polymerase chain reaction

It was noted in the late 80's that polymerase chain reaction (PCR) is an elegantly simple way to amplify a certain fragment of DNA from given starting materials (Mullis et al. 1986, 263). The replication process is due to altering temperatures - denaturation, annealing and extension happen in different degrees. A rather high temperature (+94-95 °C) is used in separating the strands of the DNA template after which the temperature is lowered (+40-72°C) and primers will base pair to complementary sequences with hydrogen bonds on the template strands. This temperature is defined carefully as it is needed to ensure that primers bind only to desired DNA sequences. The two original strands do not reanneal hence the primers are in large excess over original DNA. (Hames & Hooper 2005, 289; Thieman & Palladino 2009, 72; McPherson & Møller 2000, 5, 10.)

Finally, the temperature is set to ideal for DNA polymerase activity (~ +72°C) for efficient DNA synthesis. DNA polymerase binds to the 3' -ends of each primer adding nucleotides to synthesize a complementary strand. For optimal target DNA amplification these temperatures need to be cycled several times (25-40 cycles) depending on the method. Each cycle doubles the amount of target DNA as can be seen in figure 6. (Thieman & Palladino 2009, 72; McPherson & Møller 2000, 5, 10.)

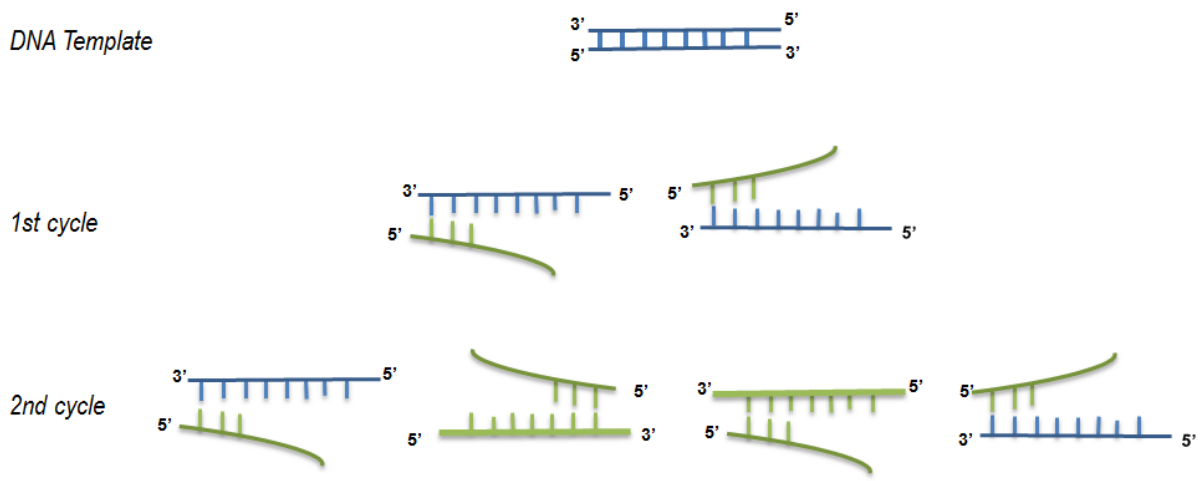

FIGURE 6: PCR amplification. Within each cycle the number of strands doubles (Based on McPherson & Møller 2000).

The designing of the primers makes the PCR truly specific. The two primers must not only to be complementary to target DNA but also not to bind together to dimers. The primers also have to have a certain guanine/cytosine percentage and almost identical annealing temperatures. (Wilson & Walker 2005, 216.) In each cycle, DNA between the two primer binding sites becomes amplified from 3' till 5' -end with four deoxynucleotides and a detailed buffer (McPherson & Møller 2000, 10).

In early cycles, the primers also try to bind to random sequences. If the sequence is not complementary to the primer they will detach and the primer reanneals elsewhere. This results from established annealing conditions such as temperature and Mg^{2+} -ion concentration. Magnesium concentration influences the specificity and efficiency of the reaction: DNA polymerase's activity is reliant upon the presence of Mg^{2+}, as it also interacts with sugar-phosphate structures of nucleic acids. (McPherson & Møller 2000, 13, 24, 68.)

Quantitative real-time -PCR (qPCR) can be considered as one of the most useful PCR applications in which the template DNA's concentration can be identified (Wilson & Walker 2005, 213-214). One real time PCR method is the 5' fluorogenic exonuclease detection system where SYBR Green, a DNA binding dye, is used in the reaction. SYBR Green attaches to the newly synthesized dsDNAs and as amplicons increase the dye's fluorescence emission can be detected. (Wilson & Walker, 2005 215-216.)

As SYBR Green being a non-specific oligonucleotide probe with a fluorescence reporter and a quencher molecule are joined in the reaction in order to detect specific amplicons. When the target sequence of the oligonucleotide probe is reached the 5' exonuclease, activity of Taq -polymerase breaks and releases the reporter from the quencher. Consequently, a signal is formed increasing the number of starting molecules in direct proportion. The progress of fluorescence can be followed in real time by detection system. (Wilson & Walker 2005, 215-216.)

qPCR is a quick, fairly simple and reliable method for determining expression levels (Wilson & Walker 2005, 216). However, to be accurately quantitative, the reaction rate should be compared to a similar template, such as the same gene. This establishes reaction sensitivity. (Gibson & Muse 2009, 237.)

3.2 Protein immunoblotting

Before the actual blotting procedure, electrophoresis needs to be performed. It is a separation technique for proteins or nucleic acids which is based on the movement of charged molecules in a permeable matrix within an electric field. In one commonly used matrix, the monomer acrylamide is polymerized forming cross-links from head to tail into long chains as bisacrylamide bridges are built to growing chain. The polymerization itself is an example of free radical catalysis which is initiated by adding ammoniumpersulphate. TEMED is used for catalysing the decomposition of persulphate ion to loose a free radical. (Switzer & Garrity 1999, 66.)

Typically, gel is constructed of two different density sections. The first layer, named stacking gel, has a lower concentration and it concentrates the sample into a tight band before entering the main separating gel. In the second section there is a high concentration of acrylamide and bisacrylamide, hence larger proteins are delayed due to frictional resistance of the gel. (Wilson & Walker 2005, 455.)

When separating proteins prior to Western blotting, polyacrylamide gel electrophoresis (PAGE) is generally performed under denaturing conditions with the presence of the anionic detergent, sodium dodecyl sulphate (SDS) (Wilson & Walker 2005, 328). Even 1,4 g of SDS can bind to a gram of protein (Reynolds & Tanford 1970, 1005) making natural charges into negative ones by detergent micelles. Glycerol is used to increase the density in the sample so that it will lay into the sample well. Bromophenol blue is a dye and constituent of the sample buffer, which migrates in front of the proteins and makes protein visualization easier. SDS-PAGE is generally applied to determine the number and size of protein chains or protein subunit chains in a protein preparation. (Switzer & Garrity 1999, 68.)

The proteins of the sample are treated with a reducing agent, such as β-mercaptoethanol, which reduces all disulfide bonds present within peptide units. Adding SDS causes disruption of noncovalent protein interactions because it binds to all regions of proteins unfolding the polypeptide chain. This sample treatment results in the total denaturation of proteins, unfolded and highly anionic polypeptide chains. The SDS coated polypeptides carry approximately one SDS molecule for two aminoacids. (Hames & Hooper 2005, 70.)

The anionic polypeptide chains are then run into a polyacrylamide gel and the appropriate buffer carries the current. Due to SDS, proteins of the sample have the same mass-to-charge ratio. (Switzer & Garrity 1999, 68.) At the end of the run, small proteins have moved furthest from the wells as larger ones have moved more slowly since they have been hindered by the cross-linking of the gel (Hames & Hooper 2005, 72).

3.2.1 Western blotting

Immunoblotting, otherwise known as western blotting, was first described in 1979 after which it has become widely used technique for analysis and detection of proteins (Towbin, Staehelin & Gordon 1979, 4350). After PAGE the proteins are transferred from gel to a membrane which is chemically inert such as nitrocellulose or polyvinylidene difluoride (PVDF). This is for further detection: specific antibodies can be attached to the membrane and the membrane can be used for chemiluminescent detection. Electrotransfer relies on the same principles as PAGE. A sandwich, described in figure 7, is assembled from the gel, membrane and electrodes to move the proteins from gel to membrane from where they can be detected. (Switzer & Garrity 1999, 291-292.)

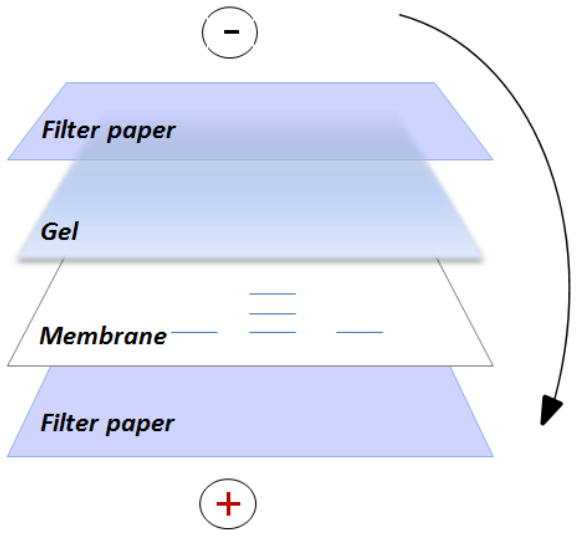

FIGURE 7: A western blotting sandwich is assembled between electrodes. The current passes from anode to cathode (Based on Switzer & Garrity 1999).

It is essential to block remaining hydrophobic binding sites on the membrane as non-specific binding of antibodies is unfavorable for the sensitivity and specificity of the assay (Wilson & Walker 2005, 457). Primary and secondary antibodies will bind nonspecifically throughout the membrane if the unoccupied spots are not blocked. It is crucial that blocking agents have a greater affinity to the membrane than the antibodies used in following steps. Blocking agents can be non-ionic detergents or proteins which include, for instance, bovine serum albumin or casein. For enhanced chemiluminescence (ECL) based detection a blocking solution is typically made of milk and Tween-20 (polyoxyethylenesorbitan monolaureate). (Switzer & Garrity 1999, 291-293.)

The protein of interest is detected from the membrane by a specific non-labeled primary antibody into which a labeled secondary antibody can bind. Secondary antibody carries the label but also possesses the mechanism to signal magnification. Secondary antibody is designated towards immunoglobulin G of the species that provided the primary antibody. (Wilson & Walker 2005, 470.)

Between the antibody incubations, the membrane is generally washed with Tween 20 which is non-ionic, nondenaturing detergent. This will disorder hydrophobic interactions of the primary antibody and other proteins which may have formed within the incubation. Secondary antibody, which is conjugated to an enzyme, recognizes the primary antibody and binds to it. The conjugate can be horseradish peroxidase (HRP), a component required for the light -producing, luminescent reaction used in detection. (Switzer & Garrity 1999, 292-293.)

Before detection the western blotted membrane is treated with signaling reagents. First reagent contains hydrogen peroxide which transforms HRP to an oxidized form as the second reagent contains luminol. Luminol is converted by HRP from reduced to oxidized form which emits light as depicted in figure 8 (p. 21). (Switzer & Garrity 1999, 293.)

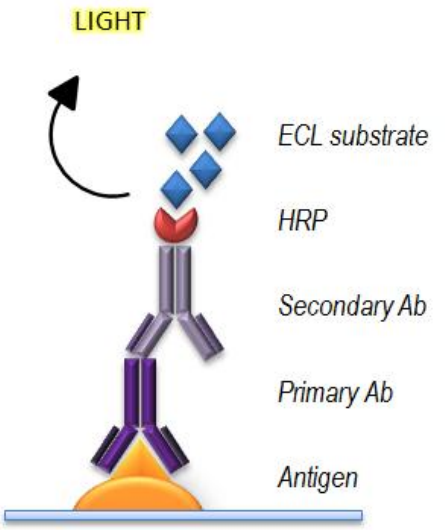

FIGURE 8: The binding of antibodies and light emission (Based on Wilson & Walker 2005).

In sensitive and rather fast enhanced chemiluminescence immunoblotting antigen-antibody -complexes are detected with peroxidase conjugated anti-immunoglobulin. This enzyme generates a peracid by dissociation of hydrogen peroxide which oxidizes the substrate. This reaction produces light which can be detected using special x-ray film. (Wilson & Walker 2005, 330-331).

3.3 Histochemistry

Histochemical methods are frequently used in identifying and localizing enzymes in tissues and cells. Firstly the fresh tissue needs to be fixed, for instance in formaldehyde, in order to terminate metabolism, prevent enzymatic decomposition and to harden the tissue. After fixation the specimen is washed and dehydrated in ascending concentration of alcohol to remove water. The organic solvents are cleared from the specimen and it is infiltrated to melted paraffin. A paraffin block is made, enclosing the specimen, which can be sliced into sections by microtome. The sections are mounted onto glass slides, paraffin is removed and the slides are stained. (Ross & Pawlina 2006, 2, 8.)

Glass slides are incubated with specific antibodies which are directed against the protein of interest. To ease the tissue examination and visualization an intense signal must be obtained by conjugating antibody with a fluorescent or enzyme label. The site of the

antibody-antigen interaction is revealed by the location of the label. (Wilson & Walker 2005, 341.)

Fluorescein absorbs ultraviolet light and emits green light which can be observed with a fluorescence or confocal microscope (Ross & Pawlina 2006, 8). When using fluorescence conjugated antibodies the fluorochrome is chosen according to the detection system. Fluorochrome -labelled antibodies are sensitive and they are easy and quick to use. Nevertheless, the fluorescein fades during prolonged viewing. In addition, autofluorescence can be problematic in some tissue components. (Wilson & Walker 2005, 344.) For reliable examination these techniques require positive and negative control antibody samples, which ensure that immunostaining truly is specific.

Usually chemical counterstaining, such as haematoxylin-eosin, is used to observe the morphology of tissue or cells (Wilson & Walker 2005, 342). Haematoxylin is a natural dye provided from a tree *Haematoxylon camperhianum*. However, it cannot be used as a dye without an incorporated mordant, like metallic salts, which operate as a bridge, enabling the staining between the tissue and the stain. (Avwioro, RE 2011, 24.)

Usually, nuclei are first stained with hematoxylin followed by eosin, staining cytoplasm and extracellular fibers nonspecifically. Eosin is an acidic dye carrying negative charge in its color component. In tissues and cells eosin reacts with ionized proteins and amino groups and other cationic groups as it binds to tissue by electrostatic linkage. (Ross & Pawlina 2006, 5-6.) One example of haematoxylin-eosin (HE) staining can be seen on figure 9, which laterally depicts a male w1118 stock fly.

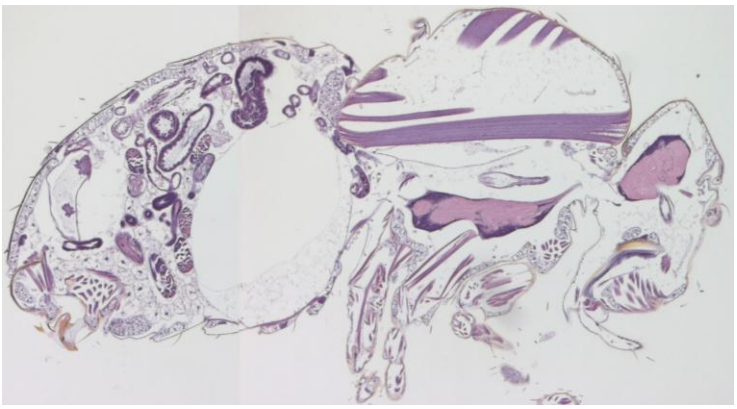

FIGURE 9: An example of HE -staining: a wild type w1118 male fly.

4 AIMS OF THE STUDY

The objective of this study was to investigate the RNA interference mediated silencing of β-carbonic anhydrase (βCA) gene in *Drosophila melanogaster*. The experimental part of the work took place in Professor Seppo Parkkila's Tissue Biology -group in the University of Tampere. The purpose of this study was to produce flies where the βCA -gene was silenced and to establish methods with which the silencing could be authenticated. Furthermore, the aim was to find out the suitable RU486 concentration for effective silencing.

The specific aims were:

1. to cross two different UAS-RNAi strains and a control strain with GeneSwitch-GAL4 -driver strain in order to gain a *Drosophila melanogaster* population in which βCA -gene could be silenced by using RU486.

2. to authenticate the silencing with three diverse methods: qPCR, western blotting and immunohistochemistry.

3. to analyze the effects of βCA -gene silencing to *Drosophila melanogaster's* phenotype.

5 MATERIALS AND METHODS

5.1 Fly crosses

Studied flies were obtained from Vienna Drosophila Stock Center and grown as stocks in the laboratory of University of Tampere in *Drosophila* Core facility. One of the stocks was a reference strain w1118: a wild type isogenic host strain which is extensively used as wild type control in *Drosophila* studies. These flies have a mutation in the white gene, which normally produces red eyes to the fly, and therefore have white eyes as seen in figure 10 (p. 25). Otherwise the w1118 line is considered as wild type and they have no insertions in their genome. (Vienna Drosophila RNAi Center 2012.) Generally the term wild type means that no identified mutant alleles are known within the stock, yet it cannot be considered as genetically homogeneous (Ashburner et al. 2005, 15).

Vienna Drosophila RNAi stocks #100233 and #38612 have been generated for βCA studies and the red color in the eyes implicates successful UAS -insertion in fly's genome. This means that as long as there are only red eyed individuals in the population the strains has its specific features at gene level. These stocks contain sequences that produce dsRNAs targeting βCA -gene under UAS control (UAS-βCA-RNAi). (Vienna Drosophila RNAi Center 2012.)

Females from stocks w1118, #100233 and #38612 were crossed with males from driver stock GeneSwitch-GAL4 as figure 10 illustrates (p. 25). For each cross, virgin females were collected to ensure the purity of crossings. Ten female virgins were gathered into a tube containing fly food (appendix 1) and 5 males were also inserted into the tube. Two of the males were adults (2 to 3 days old) and three of them were virgins (under 1 day old). Some extra yeast for better egg laying was added in the food tubes. Tubes were kept at +25 °C in an incubator with 12 h light and 12 h darkness, to ensure the best breeding activity. A CO_2 -pad was used to anesthetize the flies when sorting them.

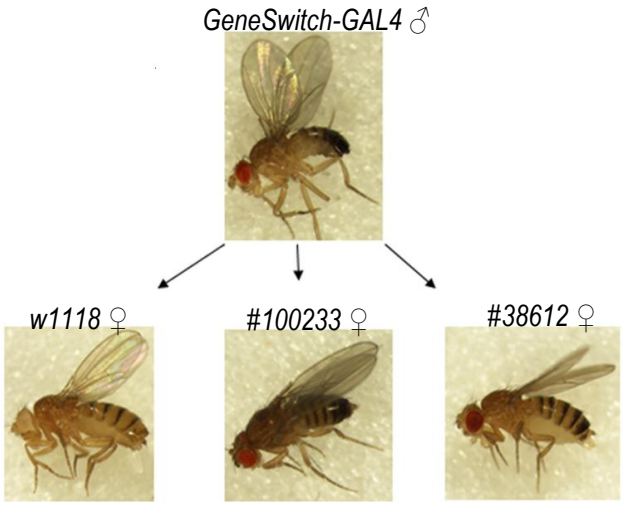

FIGURE 10: Illustrating the crosses with pictures of the fly stocks. Females w1118, #38612 and #100233 were crossed with GeneSwitch-GAL4 males.

Males were allowed to mate with the females for 72 h, and females laid fertilized eggs in the media. After 72 h the parents were flipped into new fly food tubes where fertilized females continued laying the eggs. Using this method five progeny producing tubes per cross, kept at +25 °C in 12 h light cycles, were made. From these tubes the first progeny generation was born, containing the UAS-responder sequence and inactive GAL4 protein in the same fly. This generation was flipped as one day old into special media tubes which contained certain concentration of Mifepristone (RU486; Sigma) in the media. For w1118 / GeneSwitch-GAL4 -progeny the RU486 –concentrations were 0 µM and 400 µM whereas for #100233 and #38612 / GeneSwitch-GAL4 they were 0 µM, 20 µM, 100 µM and 400 µM.

The flies were grown in RU486 containing media for five days in which period the media was once changed into fresh one. After spending five days at +29 °C degrees the flies were separated into females and males and put on dry ice (-79 °C) bed in microcentrifuge tubes. These fly samples were stored in -80 °C for RNA extraction.

5.2 RNA extraction

RNA extraction was performed using TRI Reagent Solution (Applied Biosystems) according to manufacturer's instructions. Some alterations were made to optimize the

extraction for fly tissues. The fly samples were collected as duplicates and RNA was extracted from duplicate samples separately.

Homogenization was carried out by using Pestle homogenizator tip attached to an electrical rotator. Five flies straight from -80°C degrees were put into a microcentrifuge tube containing 100 µl TRI Reagent solution and then homogenized into a fine mixture. 150 µl of TRI Reagent solutions was added, suspension was incubated for 5 min in room temperature (RT) and then centrifuged for 10 minutes in 11 300 rpm after which supernatant was collected into a new tube. Microcentrifuge tubes were kept on ice while working.

50 µl of chloroform was added into extracted supernatant and the tubes were shaken for 15 seconds after which they were incubated in RT for 5 minutes. Then suspension was centrifuged into phases at 11 300 rpm for 15 minutes. The clear aqueous phase was transferred carefully into new microcentrifuge tube and 125 µl of isopropanol was pipetted into it. This mixture was mixed at medium power for 10 seconds and incubated for 10 minutes in RT. After incubation tubes were centrifuged again at 11 300 rpm for 10 minutes in which period the RNA pellet was formed.

The supernatant was gently removed and 250 µl of 75 % ethanol, made in DEPC -treated water (appendix 2), was added into tube followed by centrifugation at 8 800 rpm for 5 minutes. After centrifugation ethanol was removed and tubes were quickly centrifuged to get any remaining ethanol to the bottom from where it was pipetted away. The washed pellet was air dried in RT for 5 minutes and then 50 µl of DEPC -treated water was added and the tube was gently mixed and incubated in heat block for 10 minutes in +60 °C degrees. During this time the pellet was dissolved into water. The solutions were frozen in -80 °C degrees for further treatments.

RNA concentration was measured using GeneQuant II RNA/DNA Calculator (Pharma Biotech) in quartz cuvette. The wavelength for this measurement was 260 nm and the sample was diluted into TE-buffer (appendix 2) 1:100. By using formula 1 the approximate value for RNA concentration was determined.

$$c\ (\mu g/\mu l) = 4 \times a \qquad \text{(FORMULA 1.)}$$

5.3 cDNA convention

RNA was transcribed into complementary DNA (cDNA) using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's instructions. All the components from table 1 were pipetted in laminar flow hood. 25 µl of diluted RNA, diluted to 2,5 µg/50 µl concentration with nuclease free water (appendix 2), was added with a gentle mix. PCR steps being: +25 °C for 10 min, +37 °C for 120 min and at +4 °C for ∞.

TABLE 1: RNA transcription into cDNA with High-Capacity cDNA Kit, reaction mix for 50 µl volumes.

Component	**µl / sample**
10x Reaction buffer	5
25x dNTPs	2
10x Random primers	5
MultiScribe™ Reverse Transcriptase	2,5
Nuclease free H_2O	10,5
Diluted RNA	25
Total	50

5.4 Quantitation with polymerase chain reaction

Real-time quantitative PCR primers were designed using Primer Express software (Applied Biosystems) for the house keeping gene RPL32 and βCA -gene. Primer sequences for RPL32 as for βCA are provided in table 2.

TABLE 2: Primers for RPL32 and βCA.

Studied gene	**Primer**
RPL32	F 5'-TTC TGC ATG AGC AGG ACC TC-3'
	R 5'-GGT TAC GGA TCG AAC AAG CG-3'
βCA	F 5'-GAC AAG GGA GCA AAT GGT CAA-3'
	R 5'-TCT ACT GTC CAT GCA GGT GAA GAA-3'

Real-time qPCR was performed using SYBR® Green PCR Master Mix kit with Applied Biosystems ABI PRISM 7000 Sequence Detection System. 96-well plate was Applied Biosystems ABI PRISM Optical Reaction Plate. The pipetted reaction mix can be seen in table 3, for both studied genes the mix was similar except for the primers. The cDNA concentration was 20 ng/µl. The standards, 0,05 ng, 0,5 ng, 5 ng and 50 ng, were made of each stocks 0 µM female samples and nuclease free water was used as control sample (NTC). The pipetting chart is described in figure 11.

TABLE 3: The reagents pipetted on the 96-well plate per well.

Component	**µl / sample**
Nuclease free H_2O	5,5
SYBR Green	7,5
Forward Primer	0,5
Reverse Primer	0,5
cDNA / Standard / Nuclease free H_2O	1
Total	**15 µl**

	1	2	3	4	5	6	7	8	9	10	11	12
A	**NTC**	**0,05**	**0,5**	**5**	**50**	0	0	20	20	100	100	400
B	**NTC**	**0,05**	**0,5**	**5**	**50**	400	0	0	20	20	100	100
C	400	400	0	0	20	20	100	100	400	400		
D	0	0	20	20	100	100	400	400				
E	**NTC**	**0,05**	**0,5**	**5**	**50**	0	0	20	20	100	100	400
F	**NTC**	**0,05**	**0,5**	**5**	**50**	400	0	0	20	20	100	100
G	400	400	0	0	20	20	100	100	400	400		
H	0	0	20	20	100	100	400	400				

FIGURE 11: An example of pipetted 96-well plate. NTC control samples are followed by standard. In rows A-D RPL32's primers used as in E-F are the βCA's primers. The light red color depicts for female samples: lighter one the first sample series and the darker one the duplicates, similarly to male samples depicted with shades of blue.

The results of qPCR were calculated using formula 2, followed by final calculation of the relative express ratio (R) using formula 3. In formula 2 the efficiency rate (E) is

calculated using the slope factor. The formula 3 determines the R based on the value of E and the deviation of crossing point (CP), the point where the fluorescence of unknown sample and a control rises above the background fluorescence. (Pfaffl 2001, 2003-2004.)

$$E = 10^{[-1/\,slope]} \qquad \text{(FORMULA 2.)}$$

$$R = \frac{(E_{target})^{\Delta CP_{target}(control-sample)}}{(E_{reference})^{\Delta CP_{reference}(control-sample)}} \qquad \text{(FORMULA 3.)}$$

5.5 Protein electrophoresis

Western blotting analyses were conducted by adapting protein extraction protocol from Kleino et al. (2008, 5414). Ten female and male #100233 / GeneSwitch-GAL4 - progeny flies of Mif -concentration 0 µM were homogenized in mixture of 100 µl lysis buffer (appendix 2) and 100 µl of 2 x Sample buffer (Sigma) using Pestle homogenizator in electronic rotator. Fly samples were incubated on ice for 45 minutes and then in a heat block for 5 minutes in +100 °C degrees. Also 5 µl of sterilized H_2O + 5 µl control sample βHisA (produced and purified by research group) was incubated in 10 µl of 2 x Sample Buffer (Sigma) in a heat block.

10 µl the standard Precision Plus Protein™ Dual Color (Bio-Rad) and 20 µl of both samples and control sample were pipetted into SDS-PAGE gel (appendix 3). 20 µl of 2x Sample buffer was pipetted to empty wells to prevent uneven protein running in the gel. The voltage was first adjusted to 100 V for 15 minutes from which on it was raised to 200 V for 45 minutes.

5.5.1 Wet blotting

After SDS-PAGE proteins were transferred onto PVDF membrane. The membrane was laid to absolute alcohol for 1 min and then to NuPage -transfer buffer (Invitrogen) for another minute and 2 filter papers and 2 wools were soaked in transfer buffer. The

transfer cassette was packed and running parameters were adjusted to 100 V and 350 mA. The run took an hour.

After the run the membrane was blocked in TBS Blotto A (Santa Cruz Biotech) in 10 ml for 30 minutes. Then the primary antibody DmβCA (produced and purified by research group, antibody produced by Innovagen) was pipetted into a blocking solution. The utilized dilution factor was 1:750 and incubation time was 1 hour in RT.

Next the membrane was rinsed once with TBST (appendix 2) and washed in TBST 4 times for 10 minutes whilst being shaken. Then the secondary antibody (Acris GmbH, R1364 HRP, anti-rabbit IgG) was added into 10 ml of blocking solution as dilution factor was 1:30 000 and incubation time of 45 min. The membrane was then washed 4 times for 10 minutes whilst being shaken in TBST.

After washes PVDF membrane was visualized using Thermo Scientific Pierce® ECL Western Blotting Substrate kit. The two components in the kit were mixed together (3,5 ml + 3,5 ml) and the membrane was incubated for one minute after which the membrane was placed to developer cassette with film. The film was let to develop for 2 and 10 minutes followed by development.

5.6 Stainings

The fly samples were fixed in 4% paraformaldehyde over night, transferred into 70% ethanol and processed in Tissue-Tek® VIP® (Sakura) into paraffin. After embedding the samples into blocks with Tissue-Tek® TEC® (Sakura) and sectioning with a microtome, the tissue samples were fixed onto Thermo Fisher SuperFrost object glasses in +37 °C degrees over night before they could be treated in xylene to erase paraffin. Next xylenes were replaced with descending alcohol series according to table 4 (p. 31). Water used in the stainings was MilliQ -systems Ultra High Purity water (UHP).

TABLE 4: Deparaffination and alcohol series. Dehydration after staining is performed in ascending order from H_2O to xylenes.

Step	**Time**
Xylene I	5 min
Xylene II	5 min
Absolute ethanol (99,9%)	3 min
94% ethanol	3 min
70% ethanol	3 min
H_2O	3 min

The haematoxylin-eosin -stain was used to examine flies morphology. The procedure is given in table 5 where deparaffination is carried out before actual staining. Also the dehydration is completed after the staining.

TABLE 5: Haematoxylin-eosin -staining procedure. Deparaffination and dehydration are performed as described in table 4.

Step	**Time**
Deparaffination	
Mayer's Haematoxylin	7 min
Running tap water	10 min
H_2O	2 min
Eosin 1%	12 sec
Running tap water	2 min
Dehydration	

5.6.1 Immunofluorescence

After deparaffination the slides were washed in 1 x PBS after UHP grade water. Blocking was performed in 0.1% BSA + 0.05% saponin in PBS solution (appendix 2) for 30 minutes in RT. After blocking the primary antibody, DmβCA was added into the blocking solution in 1:100 ratio and incubated for one hour. For negative control DmβCA pre -immune serum was used. 50 µl of both of the antibodies were pipetted in 1:100 dilution for each tissue sample.

Washes were made in blocking solution: 3 times for 5 minutes, after which the secondary antibody was added. Alexa Fluor 488 goat anti-rabbit IgG (H+L) was diluted in blocking solution and 50 µl of the antibody was pipetted for each tissue sample. The secondary antibody was incubated in the dark in RT for one hour. After incubation the slides were kept in the dark, washed in blocking solution 2 times for 5 minutes and once in 1 x PBS for 5 minutes. The slides were mounted with VectaShield® (Vector Laboratories) and kept in +4 °C degrees.

5.6.2 Immunoperoxidase

Immunoperoxidase staining was conducted using VECTASTAIN® Elite ABC -KIT with few alterations made into the procedure. After deparaffination the slides were treated with methanol + 3% H_2O_2 -solution to quench endogenous peroxidase activity followed by washes in UHP water and PBS.

Blocking solution was 10% normal goat serum in PBS and incubation time 30 minutes. The tissue samples were incubated over night in +4 °C with 1:100 dilution of primary antibody DmβCA and with negative control, pre-immune serum DmβCA. The slides were then washed 3 times with 0.1% Tween 20 in PBS for ten minutes after which the secondary antibody (Vector Laboratories, Goat Anti-Rabbit IgG) was pipetted onto slides. The incubation time for secondary antibody was 40 minutes RT followed by three 10 minute washes with 0.1% Tween 20 in PBS.

The slides were then incubated with VECTASTAIN®'s Elite ABC -reagent for 30 minutes also followed by 10 minute washes, twice with 0.1% Tween 20 in PBS and once with PBS. 50 µl of DAB -solution (Invitrogen) was placed on the tissue samples and incubated about 3 minutes. The slides were rinsed with UHP water, quickly used in Mayer's haematoxylin (Reagena) and let be under running water for ten minutes. After UHP water wash the slides were dehydrated and embedded with Entellan® (Merck).

6 RESULTS

6.1 Gene expression

The results from RNA extraction can be seen in appendix 4 where they are presented in tables 18-23. In these tables the absorbance, the ratio of (260 nm / 280 nm) and the approximate concentration for each sample are presented. RNAs from female flies were more pure than RNA from males, moreover, the RNA yield was better. Overall the purity gained from these samples, according to the ratio, was altogether sufficient.

βCA gene's expression levels, which were normalized with gene RPL32, were measured with qPCR. As summarized in table 6 (p. 34), the results from qPCR are presented in numeric form but also in figures 12-14 (p. 34–35) as graphic illustrations where on x -axis the relative expression ratio (R) is presented and in y -axis are the duplicates A and B of both sexes. In table 6 all the crossed progenies are in columns where can be seen the duplicate samples A and B of both female (F) and male (M) samples and also the RU486 concentration where flies were grown.

TABLE 6: The normalized qPCR results from each cross. F is for females, M for males, A and B are the duplicate samples.

w1118 / GeneSwitch-GAL4 -progeny		#100233 / GeneSwitch-GAL4 -progeny		#38612 / GeneSwitch-GAL4 -progeny	
Sample	**Ratio**	**Sample**	**Ratio**	**Sample**	**Ratio**
F, A, 0µM	1,00	F, A, 0µM	1,00	F, A, 0µM	1,00
F, A, 400µM	1,98	F, A, 20µM	1,54	F, A, 20µM	2,11
F, B, 0µM	1,00	F, A, 100µM	1,38	F, A, 100µM	1,68
F, B, 400µM	1,27	F, A, 400µM	1,09	F, A, 400µM	1,99
M, A, 0µM	1,00	F, B, 0µM	1,00	F, B, 0µM	1,00
M, A, 400µM	0,79	F, B, 20µM	0,88	F, B, 20µM	1,08
M, B, 0µM	1,00	F, B, 100µM	0,91	F, B, 100µM	1,13
M, B, 400µM	0,99	F, B, 400µM	0,68	F, B, 400µM	0,70
		M, A, 0µM	1,00	M, A, 0µM	1,00
		M, A, 20µM	1,09	M, A, 20µM	0,97
		M, A, 100µM	0,54	M, A, 100µM	0,64
		M, A, 400µM	0,56	M, A, 400µM	0,73
		M, B, 0µM	1,00	M, B, 0µM	1,00
		M, B, 20µM	1,34	M, B, 20µM	0,56
		M, B, 100µM	0,90	M, B, 100µM	0,54
		M, B, 400µM	0,84	M, B, 400µM	0,50

In figure 12 the effects of 0 µM and 400 µM of RU486 in the fly food are presented for w1118 / GeneSwitch-GAL4 -progeny. In the female samples the bar for 400 µM is almost twice as high as 0 µM bar. In males the alterations between these RU486 concentrations is quite slight.

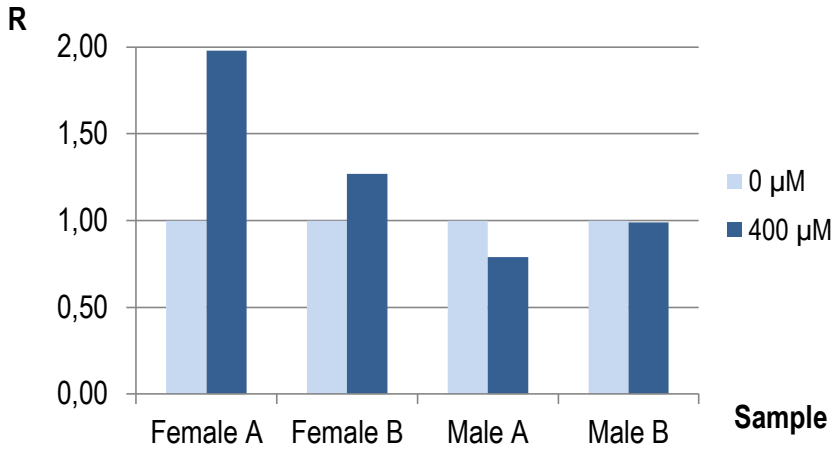

FIGURE 12: qPCR results of w1118 / GeneSwitch-GAL4 -progeny. R stands for the relative express ratio.

In figures 13 and 14 the expression levels of βCA from fly progeny with supposed βCA silencing are shown. Silencing should be dose dependent, so expression is expected to decrease upon increasing RU486 concentration. As illustrated in these figures the results show no consistent decrease towards 400 µM RU486 concentrations especially in female samples. In the males the expression level in 400 µM seems to be lower than in 0 µM.

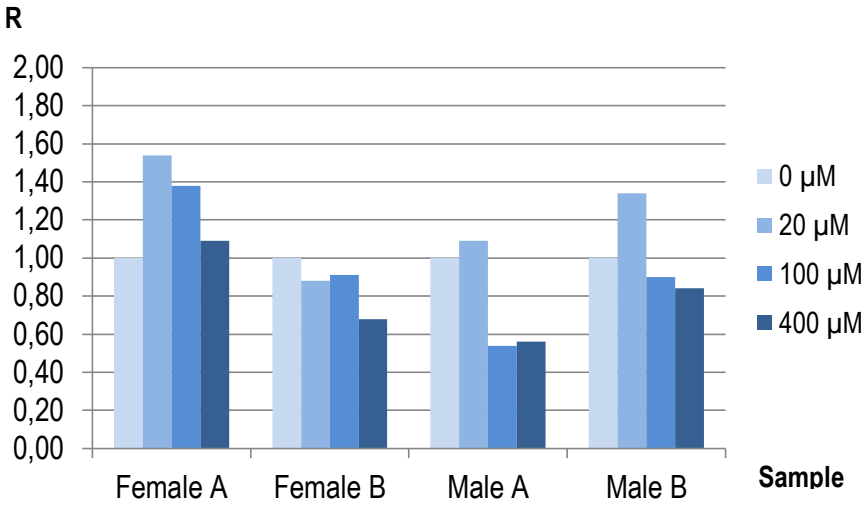

FIGURE 13: qPCR results of #100233 / GeneSwitch-GAL4 -progeny. R stands for the relative express ratio.

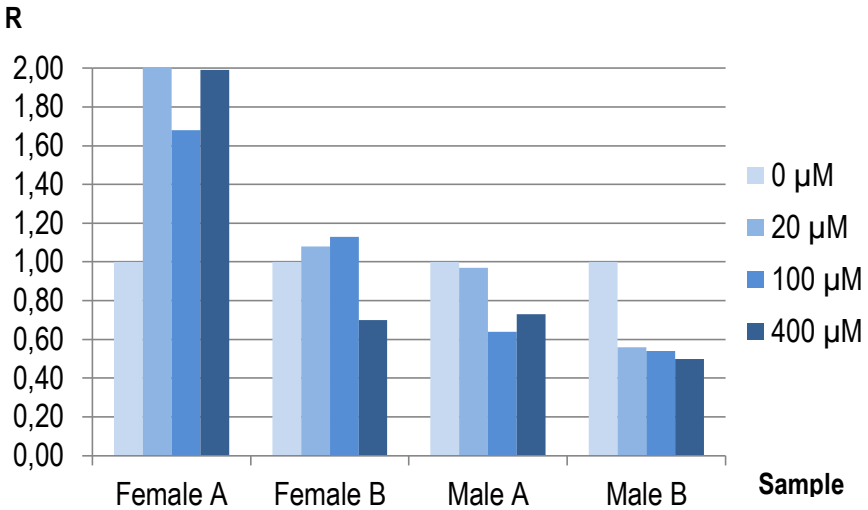

FIGURE 14: qPCR results of #38612 / GeneSwitch-GAL4 -progeny. R stands for the relative express ratio.

6.2 Immunoblotting

The functionality of the immunoblotting method was tested with #100233 / GeneSwitch-GAL4 -progeny from 0µM RU486 concentration. The idea was to examine the antibody's (DmβCA's) ability to recognize only the specific protein (βCA) from the membrane. For this the total proteins of flies were separated with SDS-PAGE. In figure 15 the first lanes in both films are female samples followed by standard and male samples. The first films (right) was developed for 2 minutes and the second (left) for 10 minutes. As the figure 15 demonstrates, bands from female samples are more intense than the male bands. It is also perceived that the antibody (DmβCA) has recognized whole variety of proteins from bands. A delicate band of βCA can be seen after 10 minutes of development in male sample as the arrow demonstrates. The concentration for separating gel was 10% and for stacking 5% (appendix 3).

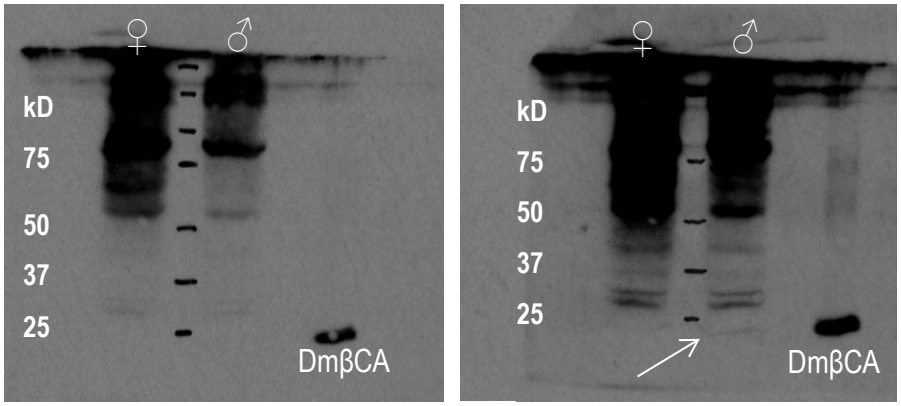

FIGURE 15: Immunoblotting results on films. The first film was let to develop for 2 minutes and the second for 10 minutes. The protein DmβCA is marked into the films.

6.3 Histological findings

Immunofluorescence was performed to examine the βCA occurrence in 0 µM and 400 µM RU486 concentrations. As can be seen from immunofluorescence images in figure 16 the results indicate that no βCA -gene silencing occurred. Alterations can be seen between positive and negative controls but in 400 µM samples the βCA activity can still be seen as fluorescence. Figure 16 (p. 37) shows female #38612 / GeneSwitch-GAL4 -progeny's abdomen. A and B are from 0 µM concentration: A being negative control

and B positive sample. C and D are from 400 µM: C depicts negative control of DmβCA and D is positive sample detected with DmβCA primary antibody.

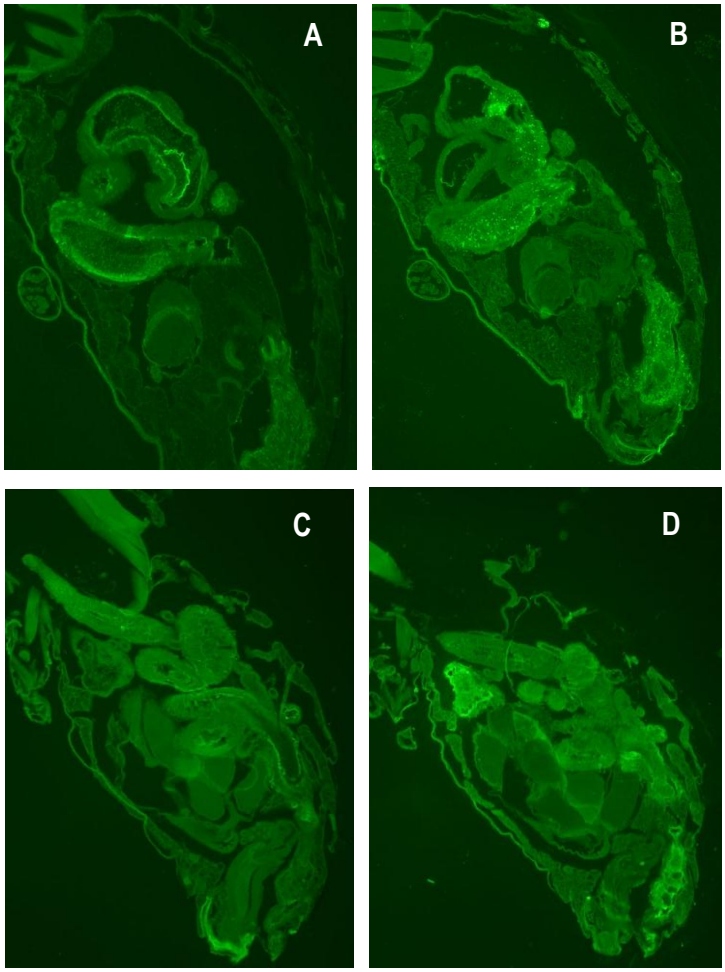

FIGURE 16: The immunofluorescence staining of female #38612 / GeneSwitch-GAL4 -progeny's abdomen.

In figure 17 (p. 38) can be seen the results of female #38612 / GeneSwitch-GAL4 -progeny stained with VECTASTAIN® Elite ABC KIT. In this immunoperoxidase stain the differences between 0 µM and 400 µM are minute. A is a negative sample from 0 µM, B is a positive sample from 0 µM, C is a negative sample from 400 µM and D is a positive sample from 400 µM.

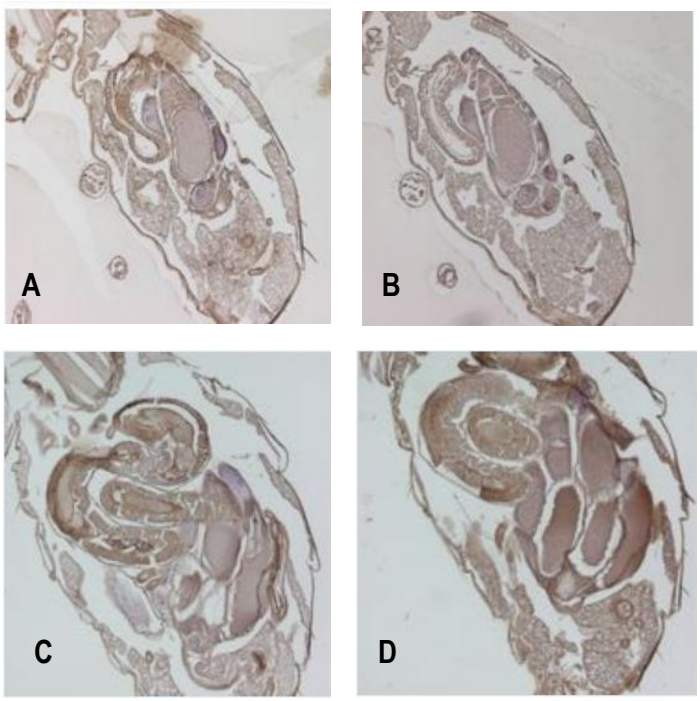

FIGURE 17: Immunoperoxidase staining of female #38612 / GeneSwitch-GAL4 - progeny's abdomen.

Figure 18 (p. 39) presents the HE -stainings of all the crossed progenies. The muscle tissues are seen as light purple stripes in fly's torso. The darker purple segments in abdomen are intestines. No clear evidence of changes between 0µM and 400 µM can be seen.

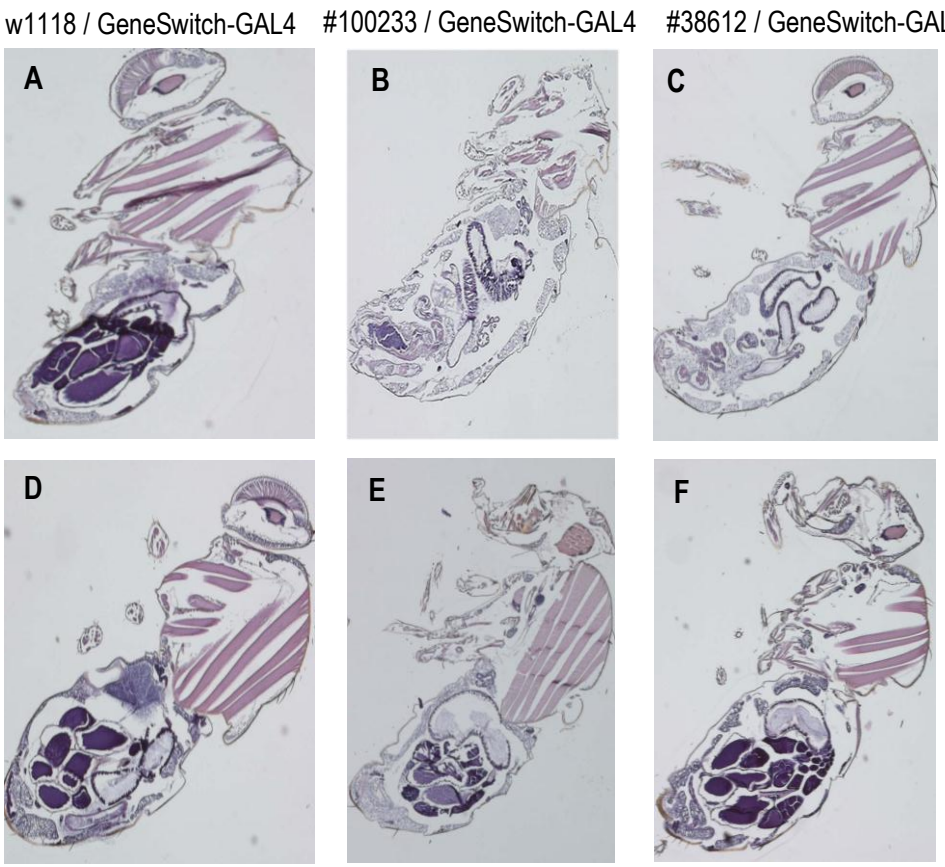

FIGURE 18: HE -staining for female flies from all crossings. A - C progenies are grown in 0 µM and D - F are grown in 400 µM RU486 fly food.

7 DISCUSSION

The objective of this Bachelor's Thesis work was to examine the effect of RNAi-based gene silencing of the β-carbonic anhydrase -gene in *Drosophila melanogaster*. The purpose was to produce *Drosophila* progeny flies where βCA -gene could be silenced, establish the methods for silencing authentication and also to find out the RU486 concentration where silencing occurs.

The first specific aim was to cross the different UAS-RNAi strains and a control strain with GeneSwitch-GAL4 -strain to gain *D. melanogaster* population which βCA -gene could be silenced. When working with flies several issues need to be taken into account. The first one is never to trust the fly stock label: when flipping dozens or hundreds of fly tubes the likelihood of human error increases. The crucial step in cross making is also the virgin collection as it needs to be absolutely certain that individual females collected are virgins. In this experiment the virgins were collected within a 12 hours timeline. Only the obvious individuals carrying certain markers were taken and the collected ones were kept for a few days in a separate tube to confirm that no larvae developed. (Ashburner et al. 2005, 18, 123.) In addition, since the flies are kept in relatively small populations mutations altering the vital parts of the genome are possible. However, the red eye color in the #100233 and #38612 stocks indicates the presence of the transgenic UAS-construct in the genome of these fly lines. (Vienna Drosophila RNAi Center 2012).

Due to the fact that GAL4-GeneSwitch is dose dependent it is necessary to discuss the used concentrations. Roman et al. (2001, 12604) reported maximum activity at 500 µM RU486 concentration as Osterwalder et al. (2001, 12598) noted that higher concentration than 280 µM (120 µg/ml) did not anymore increase the activity. Based on these publications, the 400 µM concentration used in this study should have given lowered expression levels.

The second specific aim was to authenticate the silencing with three diverse methods. Before the qPCR it was necessary to extract RNA from the flies. The yield and overall purity of the RNA extractions was satisfying, though the ratio (260 nm / 280 nm) and the concentration from female samples were more preferable than from males. This is due to the male flies being smaller in size (Weingman et al. 2003, 310). Since the size

of the male fly is only about 2/3 of the size of the female, the RNA concentration differs between sexes - however exceptions do occur in concentrations as seen in appendix 4.

In relation to the RNA concentration, some effects might be explained by the measurement appliance. The GeneQuant II RNA/DNA Calculator gave alternating results even when measuring the same sample twice. The results between two serial measurements could vary even from ±0,5 in ratio and in absorbance 0,025. These values could be measured with more precise equipment in the future to prevent alterations in RNA to cDNA transcription.

The qPCR results provided in figures 12–14 (p. 34-35) illustrate that gene expression levels between controls and samples or different RU486 concentrations were not as expected. In some cases the expression of the βCA -gene decreased as expected, although the results are not consistent. In figures 13 and 14, where the decreased expression of βCA in the UAS-RNAi/GAL4-GeneSwitch -progeny flies was expected, samples from both female groups (A and B) show rather an increased gene expression from 0 µM to 400 uM RU486 used. However, the overall results would seem to suggest that in the 400 µM RU486 concentration the gene expression might be lower than in having no RU486 at all, at least in males. The results from these figures would indicate that β-carbonic anhydrase gene silencing happens more efficiently in males than in females. However, more studies need to be performed with several samples - not only as duplicates, before definite conclusions can be drawn.

The bars should be in level with each other in figure 12 (p. 34) where w1118 / GeneSwitch-GAL4 -progenies are grown in 0 µM and in 400 µM. This is because the wild type w1118 stock has no target in its genome which GeneSwitch-GAL4 stock with RU486 could silence. In figures 13 and 14 (p. 35) the bars were expected to lower from 0 µM to 400 µM because these stocks (#100233 and #38612) have a target sequence which can be silenced when crossed to GeneSwitch-GAL4 and grown in RU486 -media. The silencing effect was estimated to be increasing with growing RU486 concentration in media (Osterwalder et al. 2001, 12598) and that might be true according to the results, except for the female A samples in #100233 / GeneSwitch-GAL4 and #38612 / GeneSwitch-GAL4 -progenies.

It was also assumed that expression level in housekeeping gene (RPL32) would not alter between the 0 µM and 400 µM concentrations when studying w1118 / GeneSwitch-GAL4 -progenies. Some differences can be detected in female samples in figure 12 (p. 34). Especially in female A -sample the expression has doubled when compared 400 µM to 0 µM. However, in the w1118 male samples the expression level alteration is almost negligible.

qPCR is known to be a very sensitive method when it comes to contamination and primer designing. Even though the results gave no evidence of primer dimers, primers might encode the wrong region from DNA (Wilson & Walker 2005, 216). It was tested in the experiment that the primers used do not match the area which has been used for RNAi construct design. However, in future experiments new primers could be designed and tried out.

Even though RNAi has been widely used it does have some limitations. It is been shown that the method does not work successfully on every gene and may not be capable of silencing the whole gene expression - even minute gene products might lead to normal phenotype. (Greenspan 2004, 50.)

One of the authentication methods was immunoblotting as to it was needed to investigate the specificity of the antibody. This was performed by studying its ability to recognize components present in sample consisting fly's total proteins. The analysis suggested that further designing should be done with the used antibody DmβCA. The antibody recognized a wide range of flies' proteins from the PVDF -membrane leading to extensive coloration of bands contrary to desired βCA band recognition. These results indicate that the specificity of the antibody was not good enough. The figure 15 (p. 36) from the developed film suggests that female bands are more intensive when compared to male bands resulting from females' size. The differentiation of bands might have been improved by using gradient gel or gel which concentration would have been greater. It could also be taken into account that using monoclonal antibody could give better results in immunoblotting.

In the future the smaller yield in RNA concentration and in purity as also in weaker bands in immunoblotting can be adjusted by taking the same amount of flies by weight or by taking few males more to compensate the females' larger size. It could also be

worth testing whether collecting selected fly tissues, where expression of βCA is predicted or shown, would give more intense signal in immunoblotting.

The third specific aim was to analyze the effects of βCA -gene silencing to *D. melanogaster*. Unfortunately, it seems to be challenging to determine the silencing effects based on the data gathered in this experiment. The fly tissue has proven to be challenging to section, stain and to examine because of the insect's chitin layer and the novelty of used antibodies in this study. The immunofluorescence method requires some adjustments since the polyclonal primary antibody seems to bind quite extensively even to negative control. In some cases the autofluorescence or the background staining can be problematic in immunostainings (Wilson & Walker 2005, 342-344). However, in this study such problems did not occur. For staining fly tissue there are only few commercial kits available suitable for *Drosophila*. In this study many different dilution factors, wash solutions and incubation times were tested which give a good basis for developing these methods even further and even more specific.

The findings suggest that silencing of the βCA -gene expression in the progeny of the UAS-RNAi flies and GAL4-GeneSwitch flies was not completely successful. However, the authentication methods were established within the experiment. For RNA extraction the process was detailed and shown to work based on the results that were collected in this study. Immunoblotting analysis and antibody were tested as they need further developing. The technique of sectioning whole flies was improved upon this study and some staining problems solved. Albeit, more studies are required in order to gain a greater comprehensive view on *D. melanogaster's* β-carbonic anhydrase gene silencing.

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APPENDICES

Appendix 1. The fly food for *D.melanogaster*.

TABLE 8: Potato-mash food.

Component	**Amount**
Water	5,5 l
Mash potato powder	200 g
Agar	50 g
Sugar syrup	250 ml
Yeast	80 g
Nipagin (10% in 96% ethanol)	42 ml
Ascorbic acid	1,25 tsb

Into boiled water yeast, syrup, mash potato powder and agar were added. Mixture was boiled up quickly, heat was reduced and mixture was cooked for 15 min. The mash was cooled to +60°C after which nipagin and ascorbic acid were added.

Appendix 2. The solutions used in experiments.

TABLE 9: DECP -water and nuclease free water.

Component	**Amount**
UHP -grade H_2O	1000 ml
DEPC	1 ml

DEPC was mixed to the MilliQ system's Ultra High Purity –water (UHP) over night and the solution was then autoclaved. For nuclease free water the DEPC -water was autoclaved once more.

TABLE 10: TE-buffer.

Component	**Amount**
TRIS	0,24 g
EDTA	0,074 g

Components were diluted into 200 ml of DEPC -water and pH was adjusted to 7.4.

TABLE 11: Lysis buffer for western blotting.

Component	**Amount**
TRIS	0,12 g
NaCl	0,82 g
1% Triton X-100	1 ml
Complete Mini Protease Inhibitor	1 tablet to 10 ml

All components except Complete Mini Protease Inhibitor (CMPI) were diluted into MilliQ -systems sterilized UHP -water and pH was adjusted with HCl to 8,0. One tablet of CMPI was added just before use to 10 ml of solution. (Kleino et al. 2008, 5414).

TABLE 12: 10 x TBS stock solution.

Component	**Amount**
TRIS	121,1 g
NaCl	175,32 g

Components were diluted into 2 liters of MilliQ -systems UHP -water.

TABLE 13: 10 x PBS stock solution.

Component	**Amount**
NaCl	80 g
KCl	2 g
KH_2PO_4	2 g
Na_2HPO_4	14 g

Components were diluted into 1 liter of MilliQ -system's UHP -water.

TABLE 14: Blocking solution: 0,1% BSA + 0,05% saponin in PBS.

Component	**Amount**
BSA	0,1 g
Saponin	0,05 g

Components were diluted into 100 ml of 1 x PBS solution.

Appendix 3. The SDS-PAGE gels and running buffer.

TABLE 15: SDS-PAGE gels

Component	**Separating gel (10%)**	**Stacking gel (5%)**
H_2O	4,0 ml	5,8 ml
30% Acrylamide, Bis Solution	3,3 ml	1,6 ml
1,5 M Tris (pH 8,8)	2,5 ml	
0,5 M Tris (pH 6,8)		2,5 ml
10% SDS	100 µl	100 µl
10% Ammonium persulfate	100 µl	100 µl
TEMED	6 µl	10 µl
Total volume	10 ml	10,11 ml

TABLE 16: SDS-PAGE running buffer stock solution (5x).

Component	**Amount**
TRIS	15 g
Glysine	72 g
SDS	5 g

Components were diluted into 1 liter of MilliQ -system UHP -water and pH was adjusted with HCl to 8,3.

TABLE 17: TRIS solutions.

Component	**0,5 M solution**	**1,5 M solution**
TRIS	121,1 g	363,3 g

Weighted amount was diluted into 2 liters of MilliQ -systems UHP -water. pH of 0,5 M solution was adjusted to 6,8 with HCl and pH of 1,5 M solution to 8,8.

Appendix 4. RNA tables.

RNA tables 18 to 23 present the absorbance, the ratio (260nm/280nm) and approximate concentration (µg/ µl) of the samples. Series A and B are the duplicates.

TABLE 18: RNA extraction results of female w1118/GeneSwitch-GAL4.

w1118 / GSGAL4	**Series**	**0 µM**	**400 µM**
Absorbance	A	0,134	0,146
	B	0,062	0,164
Ratio	A	1,94	2,077
	B	1,97	2,11
Concentration	A	0,54	0,584
	B	0,248	0,656

TABLE 19: RNA extraction results of male w1118/ GeneSwitch-GAL4.

w1118 / GSGAL4	**Series**	**0 µM**	**400 µM**
Absorbance	A	0,033	0,035
	B	0,060	0,036
Ratio	A	2,24	1,95
	B	2,08	2,07
Concentration	A	0,13	0,14
	B	0,24	0,144

TABLE 20: RNA extraction results of female #100233/ GeneSwitch-GAL4.

#100233 / GSGAL4	**Series**	**0 µM**	**20 µM**	**100 µM**	**400 µM**
Absorbance	A	0,130	0,151	0,182	0,116
	B	0,125	0,123	0,174	0,112
Ratio	A	2,08	2,10	2,11	2,12
	B	2,10	2,10	2,11	2,08
Concentration	A	0,5	0,604	0,728	0,464
	B	0,5	0,492	0,704	0,448

TABLE 21: RNA extraction results of male #100233/ GeneSwitch-GAL4.

#100233 / GSGAL4	Series	0 µM	20 µM	100 µM	400 µM
Absorbance	A	0,048	0,046	0,041	0,040
	B	0,019	0,025	0,036	0,028
Ratio	A	2,21	2,06	2,25	2,13
	B	2,00	2,14	1,87	2,16
Concentration	A	0,19	0,184	0,164	0,16
	B	0,076	0,1	0,144	0,112

TABLE 22: RNA extraction results of female #38612/ GeneSwitch-GAL4.

#38612 / GSGAL4	Series	0 µM	20 µM	100 µM	400 µM
Absorbance	A	0,11	0,174	0,214	0,154
	B	0,062	0,032	0,095	0,150
Ratio	A	2,03	2,1	2,04	2,01
	B	1,87	1,95	2,0	2,14
Concentration	A	0,43	0,696	0,856	0,616
	B	0,248	0,128	0,38	0,6

TABLE 23: RNA extraction results of male #38612/ GeneSwitch-GAL4.

#38612 / GSGAL4	Series	0 µM	20 µM	100 µM	400 µM
Absorbance	A	0,04	0,037	0,042	0,032
	B	0,061	0,054	0,054	0,049
Ratio	A	2,2	2,06	2,1	2,0
	B	2,25	2,21	2,29	2,12
Concentration	A	0,14	0,148	0,168	0,128
	B	0,244	0,216	0,216	0,196